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# Repetitive Elements in Mammalian Telomeres Suppress Bacterial DNA-Induced Immune Activation[1]

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**Bacterial DNA contains immunostimulatory CpG motifs that trigger an innate immune response capable of promoting host survival following infectious challenge. Yet CpG-driven immune activation may also have deleterious consequences, ranging from autoimmune disease to death. We find that repetitive elements present at high frequency in mammalian telomeres, but rare in bacteria, down-regulate CpG-induced immune activation. Suppressive activity correlates with the ability of telomeric TTAGGG repeats to form G-tetrads. Colocalization of CpG DNA with Toll-like receptor 9 in endosomal vesicles is disrupted by these repetitive elements, although cellular binding and uptake remain unchanged. These findings are the first to establish that specific host-derived molecules can down-regulate the innate immune response elicited by a TLR ligand.** ***The Journal of Immunology*, 2003, 171: 1393–1400.**

Deoxyribonucleic acid has multiple and complex effects on the immune system. Bacterial DNA contains immunostimulatory CpG motifs that trigger B cells, NK cells, and dendritic cells to proliferate, mature, and secrete a variety of cytokines, chemokines, and/or Ig (1–4). The cellular activation elicited by CpG motifs is mediated through Toll-like receptor 9 (TLR9)[4] (5–7). The resultant innate immune response confers a selective advantage to the host by improving resistance to a variety of infectious microorganisms (8–10).

Yet uncontrolled CpG-driven immune activation can cause harm by exacerbating inflammatory tissue damage, inducing autoimmune disease, and/or worsening shock-like syndromes (11–16). DNA from the host as well as infectious pathogens could potentially mediate such adverse events, since unmethylated CpG motifs are present in the mammalian genome (albeit at lower frequency than in bacteria, due to a combination of CpG suppression and methylation) (17–19). Recent studies indicate that mammalian DNA block, rather than exacerbate, CpG-induced immune stimulation (20–22). However, neither the sequence motif(s) responsible for this effect nor the mechanisms underlying the suppressive activity have been firmly established.

The ends of linear eukaryotic chromosomes are capped by specialized DNA-protein structures known as telomeres. In humans and mice, telomeres contain large numbers of single-stranded hexanucleotide repeats of the sequence TTAGGG (23, 24). Telomeric DNA is involved in a number of biological activities, including cell cycle regulation, cellular aging, chromosome movement/localization, and transcriptional regulation of subtelomeric genes (25–27). Whereas telomeric G-rich repeats are present at high frequency in the genomes of eukaryotes, they are extremely rare in bacteria (which lack telomeres).

This study demonstrates that TTAGGG motifs down-regulate the response to CpG DNA. Synthetic oligodeoxynucleotides (ODN) composed entirely of TTAGGG multimers reproduce this suppressive activity. They block the colocalization of CpG DNA with TLR9 within endosomal vesicles. This suppressive activity correlates with the ability of TTAGGG motifs to form G-tetrads and is abrogated by base substitutions that prevent G-tetrad formation. These findings represent the first example of a pathogen-driven innate immune response being regulated by a defined host molecule and indicate that a feedback mechanism exists to limit the tissue destruction caused by the innate immune system.

## Materials and Methods

### *Reagents*

Endotoxin-free phosphorothioate and phosphodiester ODNs were synthesized at the CBER core facility. 7-Deaza guanosine (7-DG)-modified ODNs were synthesized using the 10-camphorsulfonyl-oxaziridine oxidization protocol recommended by the manufacturer (Glen Research, Sterling, VA). Murine genomic DNA from liver and/or spleen was isolated and purified using the Wizard Genomic DNA Purification Kit (Promega, Madison, WI). *Escherichia coli* and calf thymus DNA (CT DNA) were obtained from Sigma-Aldrich (St. Louis, MO). Telomerase knockout mice (at generation 4) were produced by Dr. C. Greider (The Johns Hopkins University, Baltimore, MD) and provided by Dr. K. Hathcock (National Cancer Institute, National Institutes of Health, Bethesda, MD). All DNA obtained was repurified to eliminate endotoxin (<0.1 U/mg) (28) and was made single stranded by heat denaturation at 95°C for 5 min, followed by cooling on ice. BAL-31 (New England Biolabs, Beverly, MA) digestion of CT DNA was performed at 30°C for 90 min as recommended by the manufacturer. The enzyme was then inactivated at 65°C for 10 min. A plasmid encoding a 1.6-kb TTAGGG repeat was provided by Dr. J. Shay (University of Texas, Southwestern Medical Center, Dallas, TX). A plasmid containing nontelomeric mammalian DNA (1.2 kb) was provided by Vical (San Diego, CA.). Plasmids were amplified and digested with appropriate restriction endonucleases, and the inserts were isolated by separation on a 1% agarose followed by gel extraction.

### *Mice*

Specific pathogen-free male BALB/c mice (The Jackson Laboratory, Bar Harbor, ME) were housed in sterile microisolator cages in a barrier environment and injected i.p. with 400 $\mu$g of CpG ODN plus 200 $\mu$g of suppressive or control ODN. Spleen cells were harvested 6 h later and monitored for cytokine production over 36 h.

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[4] Abbreviations used in this paper: TLR9, Toll-like receptor 9; CD, circular dichroism; CpG ODN, unmethylated CG containing oligodeoxynucleotide; CT DNA, calf thymus DNA; 7-DG, 7-deaza guanosine; HA, hemagglutinin; KO DNA, DNA from telomerase knockout mice.

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EXHIBIT

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### *Cytokine and IgM ELISA assays*

Immulon 2 microtiter plates (Dynex Technologies, Chantilly, VA) were coated with anti-cytokine or anti-IgM Abs (BD PharMingen, San Diego CA) and then blocked with PBS/1% BSA (2). Serially diluted culture supernatants were added to these plates for 2 h. Cytokine was detected using biotinylated anti-cytokine Ab, followed by phosphatase-streptavidin (BD PharMingen), whereas bound IgM was detected using phosphatase-conjugated anti-IgM Abs (Southern Biotechnology Associates, Birmingham, AL) as previously described (2).

### *Analysis of cell surface molecule expression by FACS*

Spleen cells (2 × $10^6$/ml) were incubated with ODN for 24 h. Cells were washed, fixed with 5% paraformaldehyde for 15 min, and stained with PE-labeled anti-CD-40, anti-CD-86, and anti-ICAM-1 (BD PharMingen) for 30 min at room temperature. Cells were washed, resuspended in PBS/BSA (supplemented with azide), and analyzed by FACSort (BD Biosciences, San Jose, CA).

### *Cytokine RT-PCR*

Mice were injected with CpG and/or suppressive ODN. Total RNA was extracted from the spleens of these mice 6 h later, reverse transcribed, and amplified in a standard PCR reaction for 24 cycles using primers specific for murine IL-6, IL-12, and IFN-γ cDNA as previously described (29). PCR-amplified material was separated on 1.5% agarose gels and visualized under UV light after ethidium bromide staining (29).

### *Confocal microscopy*

HEK 293 cells stably transfected with a hemagglutinin (HA)-tagged human TLR9 construct (7) were incubated with Cy3-labeled and unlabeled ODN for 10–120 min at 37°C. Cells were washed, fixed, permeabilized, and stained with FITC-anti-HA Ab (clone 3F10; Roche, Indianapolis, IN). The subcellular localization of Cy3 and FITC signals was analyzed by laser scanning microscope (LSM5 Pascal; Carl Zeiss, Thornwood, NY).

### *Cell surface binding and internalization of ODN*

Spleen cells (2 × $10^6$/ml) were incubated with 1 μM unlabeled and/or biotinylated ODN for 10 min at 4°C (binding experiments) or for 1 h at 37°C (uptake experiments). Cells were washed and fixed, and surface-bound ODN was stained with PE-streptavidin. For internalization studies, surface-bound ODN was blocked with cold streptavidin. Cells were permeabilized and stained with PE-streptavidin and analyzed by FACSort (BD Biosciences). In some experiments the trypan blue quenching method (30) was also used to assess internalization.

### *Measurement of circular dichroism*

A Jasco J-720A spectropolarimeter was used to measure the circular dichroism of ODN (50 μg/ml in 0.1× PBS) in a range between 200–300 nm. Data are expressed as the mean peak ellipticity (millidegree per absorbance) of 5–10 readings per sample in the 260–270 nm range.

### *Statistical analysis*

In vitro assays were performed in triplicate on at least three different spleen cell preparations. Statistical significance was evaluated using Student's *t* test. Correlation analysis is computed by linear correlation analysis between circular dichroism (CD) data vs percent suppression.

## Results

### *Telomeric regions of mammalian DNA suppress CpG-induced immune activation*

The ability of bacterial and mammalian DNA to trigger an innate immune response was evaluated by monitoring the production of IL-12 by murine spleen cells in vitro. As shown in Fig. 1, single-stranded bacterial DNA and CpG ODN stimulated IL-12 production, whereas single-stranded mammalian DNA suppressed this activity ($p < 0.01$).

In an effort to identify the sequence motif(s) or structural element(s) responsible for the observed suppression, the mammalian DNA was digested with a variety of restriction enzymes. Treatment with Bal-31 (which selectively removes bases from the telomere-containing ends of the chromosome) (31) significantly reduced suppressive activity (Fig. 1*b*). Nearly 5-fold more BAL-31-digested DNA was required to achieve a 50% reduction in CpG-induced cytokine production than native DNA (data not shown).

To pursue this finding, DNA was isolated from plasmids encoding either telomeric or nontelomeric mammalian DNA. The 1.6-kb fragment of pure telomeric DNA suppressed CpG-induced IL-12 production significantly more effectively than an equivalent amount of nontelomeric DNA ($p < 0.01$; Fig. 2*a*). Since some suppression was mediated by the nontelomeric fragment, we postulated that telomeric DNA accounted for much, but not all, of the

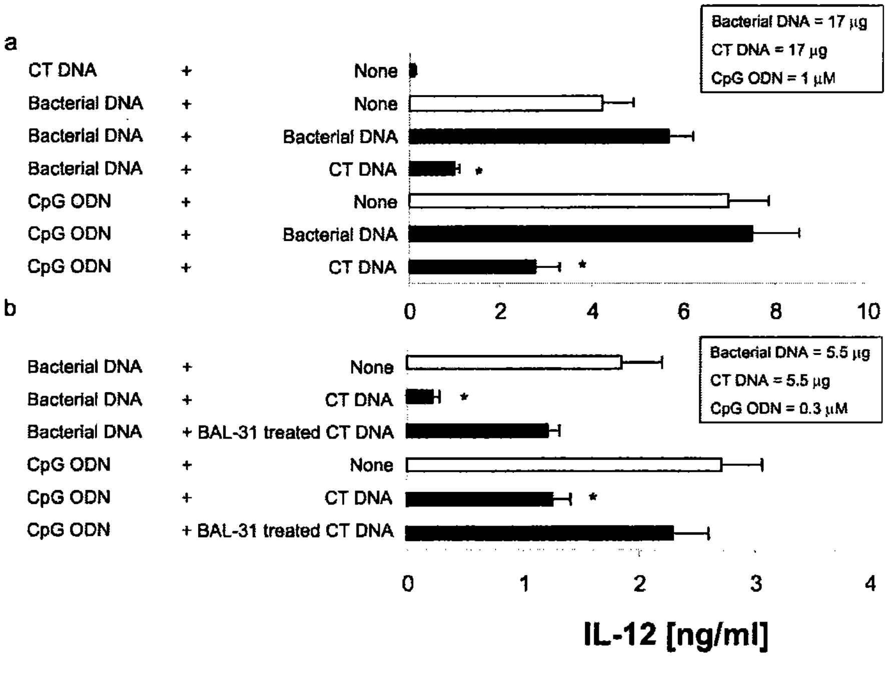

**FIGURE 1.** Mammalian DNA suppresses CpG DNA induced IL-12 production. *a*, BALB/c spleen cells were incubated in vitro for 36 h with 17 μg of single-stranded calf thymus (CT) or bacterial DNA and/or 1 μM CpG ODN (TCAACGTTGA, a sequence present in mammalian DNA). *b*, CT DNA was digested with Bal-31 to remove telomeric ends. Spleen cells were then stimulated in vitro with 5.5 μg of single-stranded bacterial or mammalian DNA and 0.3 μM CpG or control ODN. IL-12 levels in culture supernatants were measured by ELISA. All assays were run in triplicate. The experiment was repeated with similar results. *, Significantly different from cultures stimulated with CpG DNA alone, $p < 0.01$.

suppressive activity of mammalian DNA. To test this hypothesis, DNA was purified from normal and generation 4 telomerase-deficient knockout (KO) mice (telomere length is significantly reduced in the KO animals) (25). Five- to 10-fold more DNA from telomerase deficient mice was required to achieve 50% suppression than normal DNA ($p < 0.01$; Fig. 2*c*). Taken together these results indicate that telomeres are responsible for a majority of the suppression mediated by mammalian DNA.

### *G-tetrad-forming telomeric TTAGGG repeats mediate suppression*

Telomeres contain large numbers of highly conserved, single-stranded, guanosine-rich hexameric motifs (TTAGGG in mice and humans) (27). ODNs were synthesized that contained one or more of these motifs. A 6-mer ODN composed of a single TTAGGG did not reduce CpG-induced cytokine production (Fig. 3*a*). However, the same motif incorporated into a longer control ODN (≥8 bp) was suppressive. ODNs containing multiple TTAGGG motifs were even more suppressive (Fig. 3*a*). TTAGGG multimers inhibited a broad range of CpG-dependent immune stimulation in a dose-dependent fashion, including the up-regulation of costimulatory molecules on APCs, IgM production by B cells, and NO release from macrophages ($p < 0.001$; Fig. 3*a* and Table I). Phosphodiester and phosphorothioate TTAGGG multimers at equimolar concentrations were equivalent in their ability to block CpG-induced immune activation both in *cis* and in *trans* (i.e., they inhibited the stimulatory activity of CpG motifs

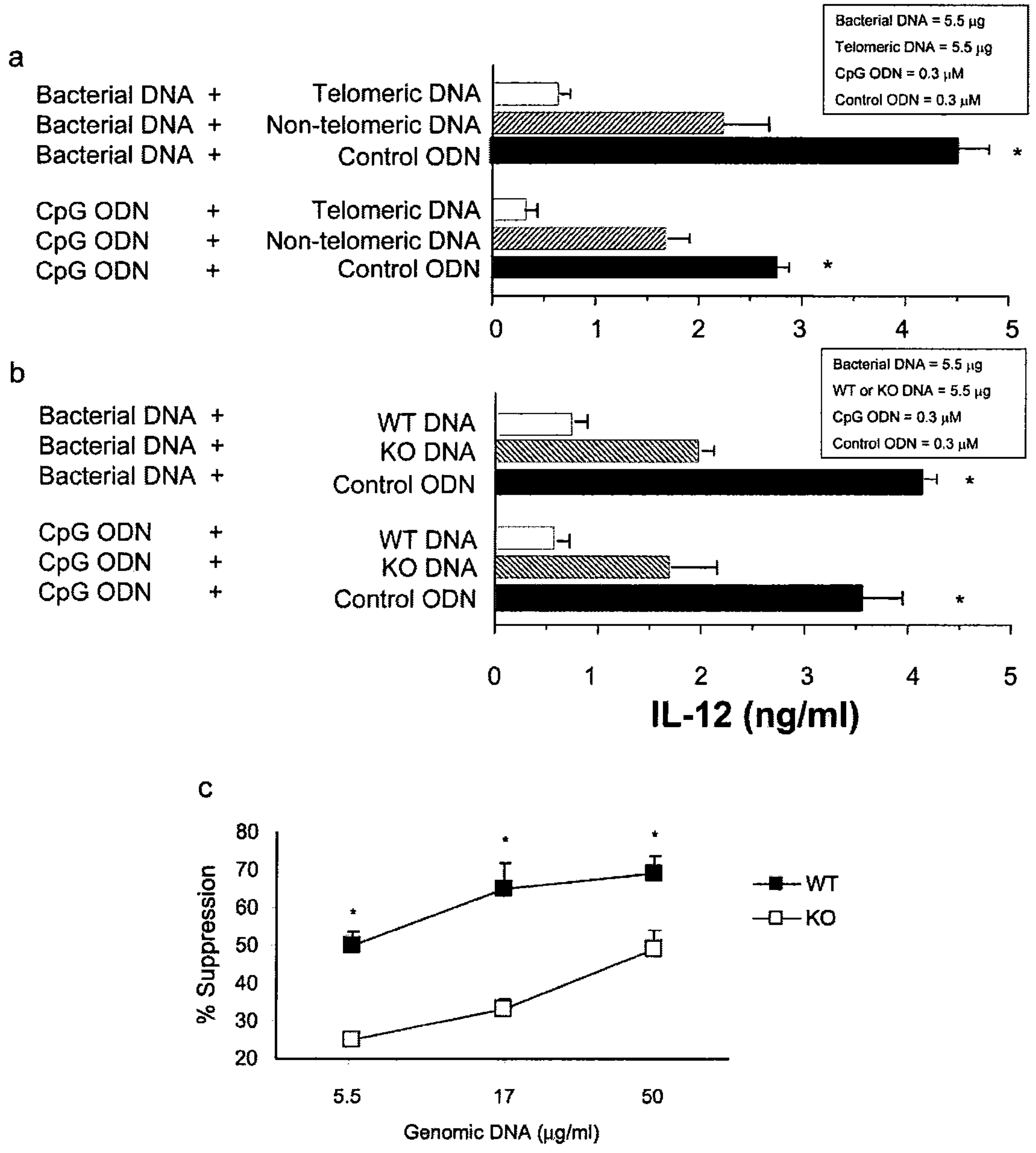

FIGURE 2. Telomeres mediate the suppressive activity of mammalian DNA. BALB/c spleen cells were stimulated in vitro for 36 h with CpG ODN (0.3 μM) or single-stranded bacterial DNA (5.5 μg/ml). Data show the change in IL-12 production (monitored by ELISA) associated with the addition of various types of DNA to CpG-stimulated cultures. All assays were run in triplicate, and the experiment was repeated with similar results. *a*, Effect of single-stranded telomeric or nontelomeric DNA (5.5 μg/ml) on IL-12 production. *b*, The effect of single-stranded DNA from generation 4 telomerase KO or wild-type (WT) mice (5.5 μg/ml) was evaluated. *c*, The suppressive activity of the DNA evaluated in *b* was monitored over a range of concentrations. *, Significantly different from cultures stimulated with CpG DNA alone, $p < 0.01$.

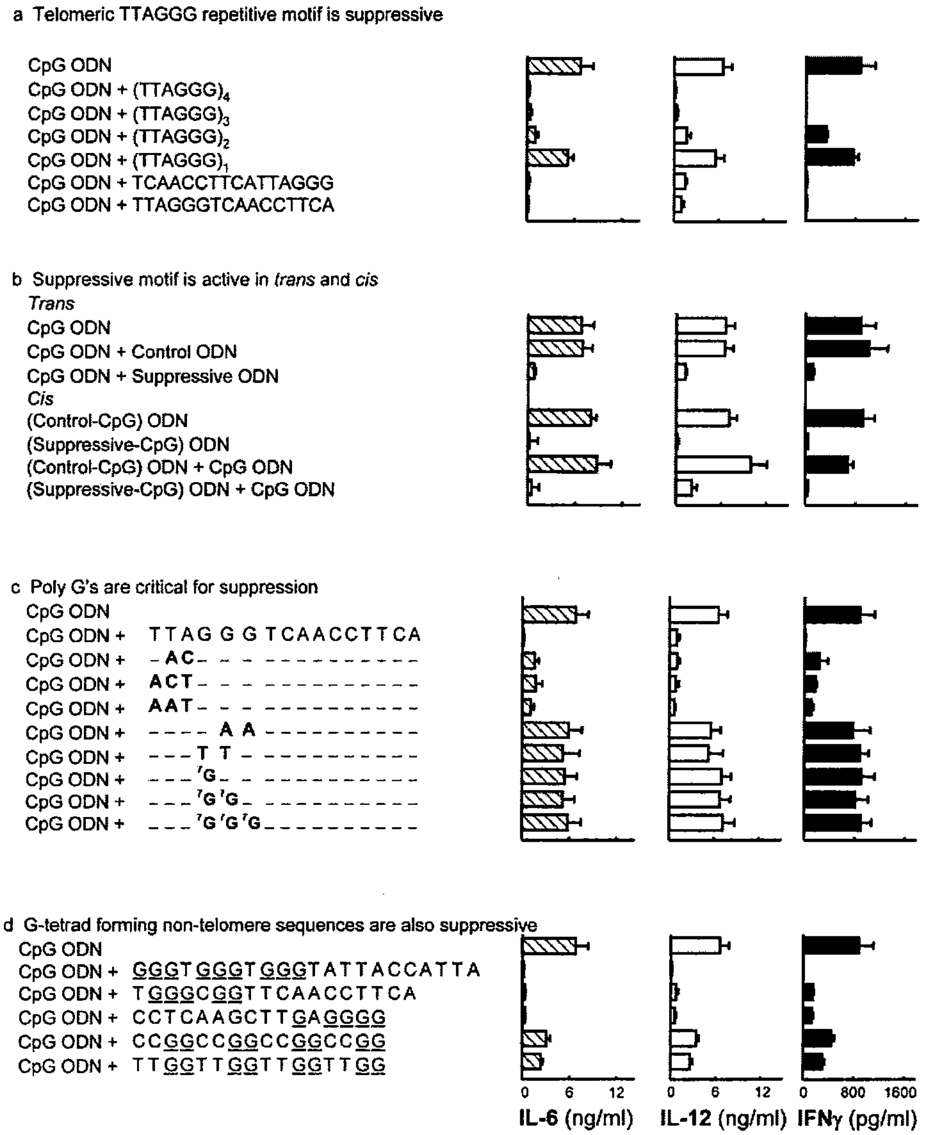

**FIGURE 3.** Factors contributing to the suppression of CpG-induced immune activation. *a*, BALB/c spleen cells were stimulated in vitro for 36 h with 1 μM CpG ODN and/or 1 μM suppressive ODN $(TTAGGG)_4$. IL-6, IL-12, and IFN-γ levels in culture supernatants were quantitated by ELISA. *b*, For studies of the *trans* effect, spleen cells were stimulated with 1 μM CpG ODN (TCAACGTTGA) plus 1 μM of either a control (TCAACCTTCA) or a suppressive $(TTAGGG)_2$ ODN. For studies of the *cis* effect, single ODNs were synthesized that contained a control (TCAACCTTCA) or suppressive $(TTAGGG)_2$ motif coupled to a stimulatory motif (TCAACGTTGA). *c*, Tetrad formation was monitored by peak ellipticity of the ODN at 260–270 nm (millidegree per absorbance). $^7$G represent a 7-DG substitution. *d*, G-rich (underlined bases), tetrad forming ODNs (1 μM) were coincubated with CpG ODN (TCAACGTTGA) or control ODN (TCAACCTTCA), and cytokine levels in culture supernatants were quantitated by ELISA. All results represent the average of at least two independent experiments performed in triplicate. Studies involving the first two ODNs, shown in *d*, and various TTAGGG multimers show that similar levels of suppression were obtained using either phosphodiester or phosphorothioate ODN.

located on the same or a different strand of DNA; Fig. 3, *b* and *d*, and data not shown).

To identify the bases contributing to this suppressive activity, the TTAGGG motif was systematically modified. Substitutions outside the telomere-derived region had no effect on suppression (data not shown). Replacing the TTA bases also had little effect on suppressive activity (Fig. 3*c*). In contrast, replacing two or more G's in the TTAGGG motif significantly reduced the ability of ODN to block CpG-induced immune activation ($p < 0.02$; Fig. 3*c*). These results suggested that suppression was mediated by either the GGG sequences or the two-dimensional structure they conferred on the ODN.

To differentiate between these alternatives, the G's were replaced with 7-DG base analogs. These 7-DG analogs did not alter the base sequence of the ODN, but did prevent Hoogsteen hydrogen bonding between guanosines, thereby inhibiting the formation of G-tetrads (32, 33). The resultant loss in tetrad structure is reflected by a decrease in the CD of the molecule (33). ODNs capable of forming G-tetrads typically have CD values >2, while those without such high order structure have ellipticity values <1.4 (Fig. 4). Substituting a 7-DG analog for any of the G's in the TTAGGG motif significantly reduced the ability of ODN to form a G-tetrad and its ability to mediate suppression ($p < 0.001$; Figs. 3*c* and 4).

To confirm that G-tetrad formation was critical to suppression, ODNs were synthesized that lacked the TTAGGG motif, but still formed G-tetrads. These novel ODNs significantly suppressed CpG-induced immune stimulation ($p < 0.001$; Fig. 3*d*). Consistent

Table I. *Effect of suppressive ODN on CpG DNA-mediated immune activation*[a]

| | % Inhibition | | | |
|---|---|---|---|---|
| | Suppressive:CpG ODN Ratio | | | |
| | 1:3 | 1:1 | 3:1 | 10:1 |
| CD40 | 21.5 ± 2.3 | 42.0 ± 5.0 | 79.3 ± 7.1 | 85.2 ± 5.3 |
| CD86 | 28.9 ± 6.3 | 36.5 ± 4.1 | 52.6 ± 2.3 | 66.2 ± 4.6 |
| ICAM-1 | 32.2 ± 2.1 | 38.7 ± 5.2 | 56.6 ± 4.3 | 74.2 ± 6.1 |
| IL-6 | 50.2 ± 6.8 | 78.4 ± 6.5 | 91.5 ± 4.6 | 97.3 ± 4.2 |
| IL-10 | 48.3 ± 5.6 | 72.1 ± 5.5 | 95.4 ± 3.1 | 96.4 ± 2.5 |
| IL-12 | 59.4 ± 7.2 | 83.1 ± 4.9 | 90.6 ± 3.1 | 95.7 ± 2.9 |
| IFNγ | 41.0 ± 2.6 | 72.6 ± 4.0 | 87.2 ± 3.8 | 96.6 ± 2.7 |
| IgM | 44.0 ± 4.8 | 56.3 ± 8.4 | 73.5 ± 5.2 | 85.0 ± 6.2 |
| NO | 36.4 ± 3.0 | 49.5 ± 6.2 | 81.6 ± 6.7 | ND |

[a] Spleen cells were incubated with 0.1-3 μM CpG and/or suppressive ODN. Levels of CD40, CD86, and ICAM-1 expression (MFI) were determined by FACS after 24 h. IL-6, IL-10, IL-12, IFN-γ, and IgM levels in culture supernatants were determined by ELISA. The Griess method was used to detect NO in culture supernatants after 48 h. % Inhibition was calculated by the formula: (1 − [(activation by CpG DNA + suppressive ODN) − (background)/(activation by CpG DNA + control ODN) − (background)]) × 100. Suppressive ODN, TTAGGG$_4$; CpG ODN, GCTAGACGTTAGCGT. ND, Not determined. Note that all data points represent statistically significant suppression of CpG-induced immune stimulation ($p < 0.05$).

with results involving TTAGGG multimers, 1) substituting a 7-DG in these ODNs abolished both G-tetrad formation and suppressive activity, and 2) these ODNs were equally active in phosphodiester and phosphorothioate form (data not shown). As shown in Fig. 4, there was a consistent correlation between the suppression mediated by an ODN and its circular dichroism ($r = 0.832$).

### *Suppressive ODN down-regulate CpG-induced immune activation in vivo*

Unrelieved or overexuberant stimulation of the innate immune system by CpG DNA may cause tissue damage, autoimmune disease, or even death (11–13, 34, 35). We postulated that the repetitive elements in telomeric DNA might prevent endogenous CpG motifs from chronically activating the host's immune system. Studies were therefore conducted to examine whether suppressive DNA was active in vivo.

BALB/c mice were injected i.p. with 400 μg of CpG DNA. Consistent with previous reports (2), this CpG DNA systemically activated the host's immune system, as manifest by increased cytokine production (Fig. 5, mRNA or protein level) and MHC class II expression (1, 17). When 200 μg of control ODN was coadministered with the CpG DNA, no interference in immune activation was observed. In contrast, coadministering 200 μg of suppressive ODN with CpG DNA reduced cytokine levels by >75% and down-regulated MHC expression by >50% ($p < 0.01$; Fig. 5, *a* and *b*, and data not shown). Of note, inhibition was observed when the suppressive sequence was expressed on the same or a different fragment of DNA carrying the CpG motif (data not shown).

### *Suppressive ODN block the colocalization of CpG DNA with TLR 9 in endosomal vesicles*

The mechanism by which suppressive ODN inhibit CpG-induced immune activation was explored. Suppressive ODN did not significantly reduce LPS or mitogen-induced cytokine or Ig production (data not shown), demonstrating that they were neither toxic nor nonspecifically immunosuppressive.

| ODN Structures | Ellipticity (mdeg/abs) |
|---|---|
| A- (TTAGGG)$_4$ | 7.1 |
| B- GGGTGGGTGGGTATTACCATTA | 4.9 |
| C- (TTAGGG)$_3$ | 4.8 |
| D- CCTCAAGCTTGAGGGG | 4.6 |
| E- TGGGCGGTTCAACCTTCA | 4.1 |
| F- TTAGGGTCAACCTTCA | 3.4 |
| G- TTGGTTGGTTGGTTGG | 3.2 |
| H- CCGGCCGGCCGGCCGG | 2.8 |
| I- (TTAGGG)$_2$ | 2.6 |
| J- (TTAGGG)$_1$ | 1.2 |
| K- TTA$^7$GGGTCAACCTTCA | 1.0 |
| L- TTA$^7$G$^7$G$^7$GTCAACCTTCA | 0.8 |

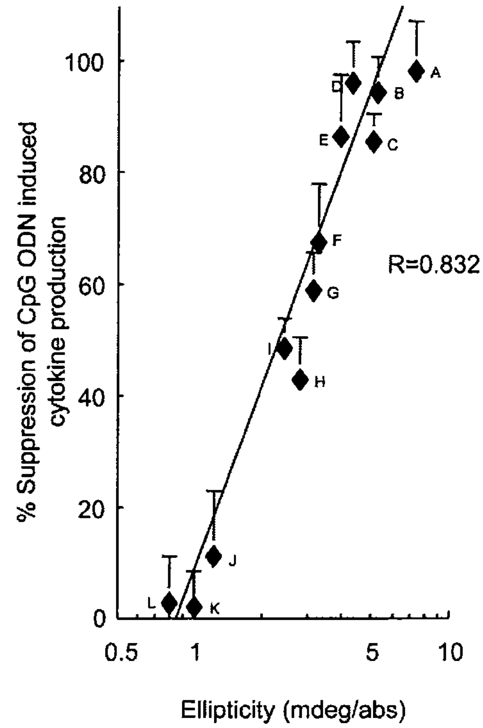

FIGURE 4. ODN tetrad-forming ability correlates with suppressive activity. G-tetrad formation and suppressive activity of multiple ODNs (structures from *A–L*) were monitored. $^7$G, 7-DG substitution. Results show the correlation between the percent suppression of CpG-induced cytokine production (average suppression of IL-6, IL-12, and IFN-γ production in Fig. 3) vs ellipticity. The percent suppression was calculated by the formula: {1 − [(activation by CpG DNA + suppressive ODN) − (background)/(activation by CpG DNA + control ODN) − (background)]} × 100. The correlation coefficient was determined by linear analysis.

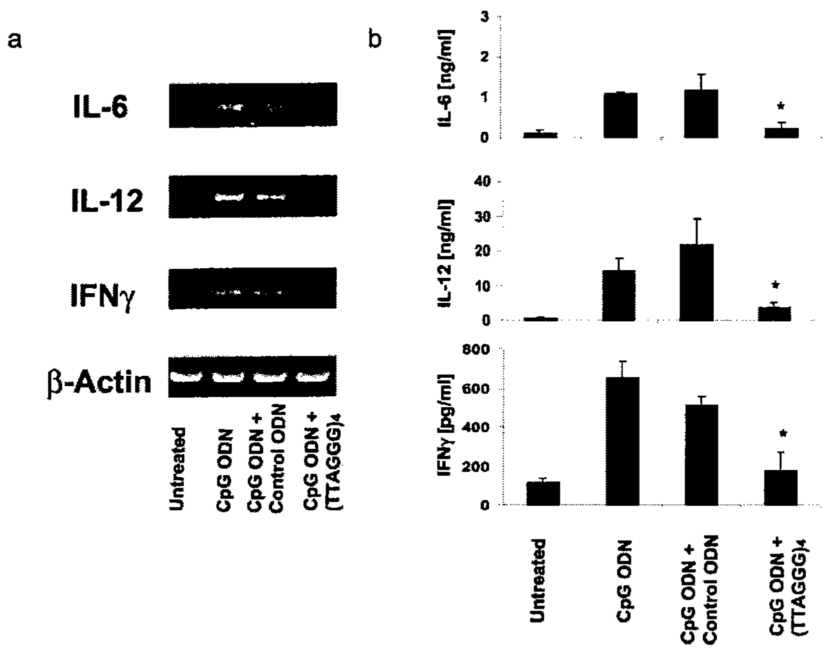

**FIGURE 5.** In vivo effects of suppressive ODN. BALB/c mice were injected i.p. with 400 $\mu$g of CpG ODN (GCTAGACGTTAGCGT) plus 200 $\mu$g of suppressive (TTAGGG)4 or control ODN (GCTA GATGTTAGCGT). *a*, Cytokine mRNA levels were monitored by RT-PCR of spleen cells isolated 6 h after treatment. *b*, Spleen cells from treated mice were cultured ex vivo for 36 h without further stimulation. Cytokine levels were quantitated by ELISA. Data represent the average ± SD of three mice per group, analyzed independently. *, Significantly different from animals stimulated with CpG DNA plus control ODN, $p < 0.01$.

FITC-labeled ODNs were used to monitor the interaction between suppressive and CpG DNA. As shown in Fig. 6*a*, suppressive ODN did not block the binding or uptake of CpG ODN by immune cells. HEK cells were then transfected with a construct encoding TLR9 tagged with an HA epitope (TLR9-HA) (29). Cells transfected with HA-TLR9 are readily visualized by addition of fluorescein-labeled anti-HA Ab (Fig. 6*b*, green) and respond to stimulation by CpG, but not control ODNs (29). Consistent with previous reports, Cy3-labeled CpG ODN (red) colocalizes with TLR9 in endosomal vesicles (yellow), an interaction that triggers an increase in both the number and size of these vesicles (Fig. 6, arrows) (29). Whereas control ODN had no effect on the interaction between TLR9 and CpG DNA (Fig. 6*d*), suppressive ODN blocked both the colocalization of CpG ODN with TLR9 in endosomal vesicles and the expected increase in the size and number of such vesicles (Fig. 6*e*).

## Discussion

The innate immune response triggered by CpG motifs in bacterial DNA can improve host survival following pathogen challenge (9, 36, 37). Yet unchecked stimulation of the innate immune system can cause tissue damage, autoimmune disease, and even death (11–13, 34, 35). This work establishes that repetitive telomeric motifs can down-regulate CpG-induced immune responses. TTAGGG multimers inhibited all forms of CpG-induced immune activation, including the stimulation of B lymphocytes, dendritic cells, and macrophages (Table I). Moreover, TTAGGG multimers were suppressive both in *cis* and in *trans*, consistent with an ability to inhibit both endogenous and foreign CpG DNA (Fig. 3*c*). The suppressive activity of these TTAGGG motifs correlated with their ability to form G-tetrads.

Several observations support the conclusion that telomeres are responsible for much of the suppressive activity of mammalian DNA. First, digestion of these telomeres with BAL-31 reduced the suppressive activity of mammalian DNA by 5- to 10-fold (Fig. 1). Second, DNA from telomerase KO mice was 10-fold less effective at blocking CpG-induced immune activation than DNA from normal mice (Fig. 2). Finally, pure telomeric DNA was significantly more suppressive than DNA from a nontelomeric region of the genome (Fig. 2).

Additional study is needed to establish whether telomeric DNA is suppressive under physiologic conditions. Individual mammalian cells contain >10,000 phosphodiester TTAGGG elements, and some of these exist in single-stranded form (27). Thus, the type of TTAGGG multimer found in the current work to mediate suppression (Fig. 3) would be present at high local concentrations when released following the death of host cells. The observation that TTAGGG ODN inhibited immune activation at one-third the molar concentration of CpG DNA (Table I) further suggests that such suppression is biologically relevant.

Suppressive activity correlated with the ability of TTAGGG motifs to form G-tetrads (Fig. 4). Nucleotide substitutions that interfered with G-tetrad formation abrogated suppressive activity (Fig. 3*c*). In this context, the frequency of G-tetrad-forming sequences is significantly higher in the genomes of mammals (such as mice and humans) than bacteria (such as *E. coli*, *Klebsiella*, *Bacillus subtilis*, and *Staphylococcus aureus*). Indeed, even the nontelomeric DNA used in this study had 21 regions containing ≥4 polyG runs (Fig. 2*a*).

Mammalian telomeres are not the only sequence motifs with suppressive properties. Pisetsky et al. (21) reported that ODNs containing long runs of poly(A), T, C, or G can inhibit CpG-dependent IL-12 production in vitro. However, such motifs are quite rare in mammalian DNA. Kreig et al. (38) identified neutralizing sequences in adenovirus DNA that reduced the host's ability to mount an immune response against virus-infected cells. These neutralizing sequences are GC rich and tend to contain methylated cytosines (21). Our analysis of these motifs indicates that 1) the relevant adenoviral sequences form G-tetrads; and 2) the methylation status of the cytosines is irrelevant, since cytosines can be replaced with thymidines with no loss of suppressive function (Fig. 3*d* and data not shown). These findings are consistent with tetrad formation, rather than GC sequences or modification of C, being critical to suppressive activity. A recent report by Stacey et al. (39) also found that G-rich motifs can be suppressive, and that the methylation status of cytosines is unrelated to suppressive activity. Thus, while the present work is not the first to identify motifs that can down-regulate CpG-mediated immune activation, it is the first to identify the critical element(s) in the mammalian genome responsible for such suppression.

Studies were undertaken to clarify the mechanism by which suppressive DNA blocked immune activation. Several possibilities

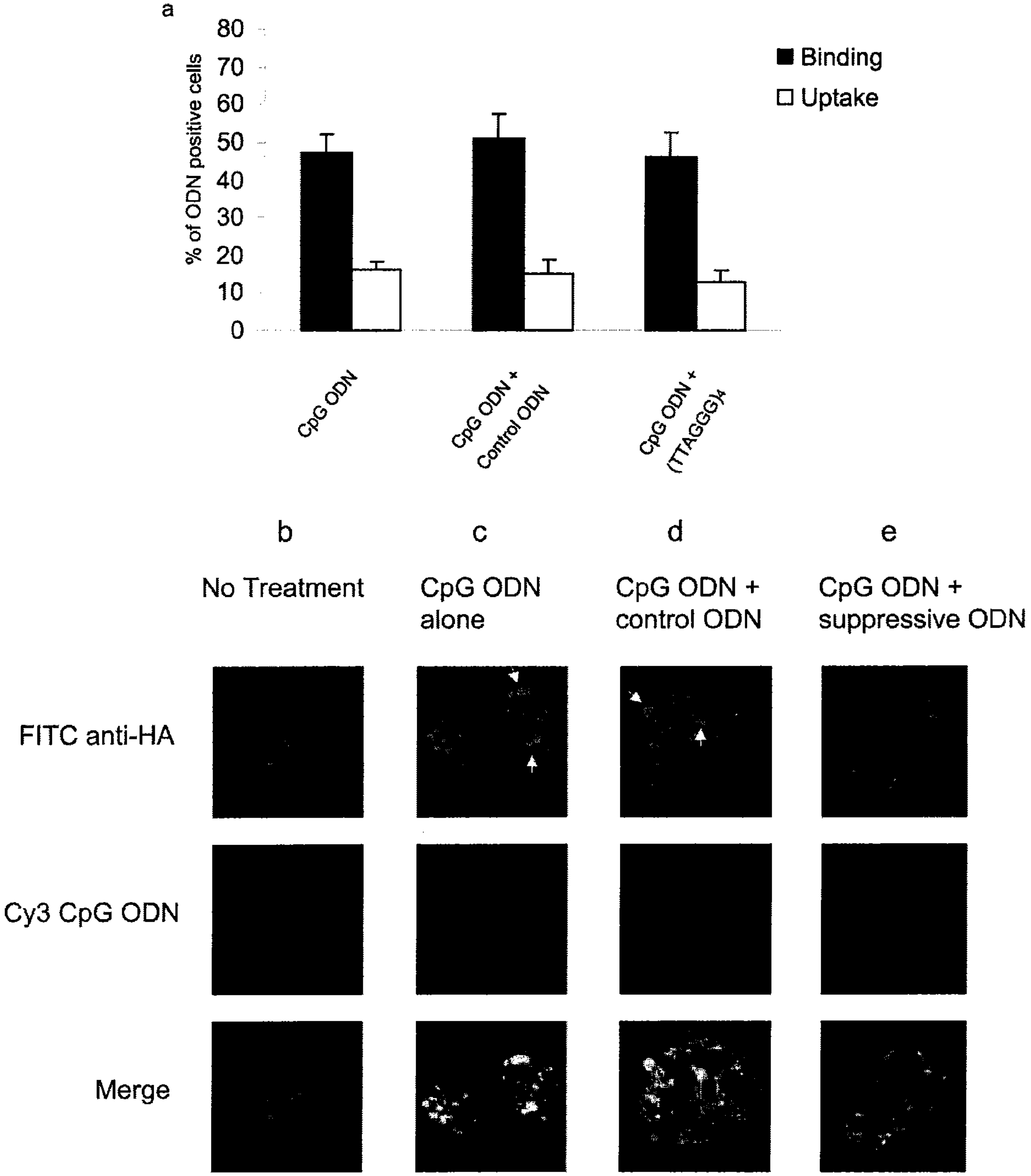

**FIGURE 6.** Effect of suppressive ODN on the uptake and localization of CpG ODN. *a*, BALB/c spleen cells were incubated with 1 μM CpG ODN (GCTAGACGTTAGCGT) labeled with fluorescein (to detect cell surface binding) or biotin (to detect internalization) plus an equal amount of unlabeled $(TTAGGG)_4$ suppressive or control ODN (GCTAGATGTTAGCGT). The number of cells that bound or internalized CpG ODN was determined by FACS analysis. Similar results were obtained using several different CpG, control and suppressive ODN. *b–e*, HEK 293 cells transfected with TLR9-HA were stained with anti-HA-FITC (green, *upper panels*) and incubated with 1 μM Cy3-labeled CpG ODN (red, *middle panels*) for 2 h. The colocalization of TLR9 and CpG ODN is shown in the *lower panels* (yellow). The interaction of TLR9 with CpG ODN and the associated increase in the number and size of endosomal vesicles (arrows) were reduced by the addition of unlabeled suppressive (*e*) but not control (*d*), ODN.

were eliminated: suppressive ODN were not toxic, they did not inhibit mitogen or LPS-induced immune activation, and they did not prevent the binding or uptake of CpG DNA by immune cells (Fig. 6) (40). Rather, they interfered with the maturation of endosomal vesicles and the colocalization of CpG DNA with TLR9 in these vesicles. The ability of suppressive motifs to block this ligand-receptor interaction is consistent with other studies showing that very early events in CpG mediated signaling (NF-κB translocation) are inhibited by suppressive ODN (40, 41).

The magnitude and duration of adaptive immune responses are regulated by both stimulatory and suppressive signals (42, 43). Current findings provide evidence that innate immune responses may be similarly regulated by host-derived molecules. In vivo, suppressive ODN blocked CpG-induced cytokine production by

>75% (Fig. 5). It is interesting that unmethylated CpG motifs present at high frequency in bacterial, but not mammalian, DNA stimulate the innate immune system, whereas telomeric repeats present at high frequency in mammalian, but not bacterial, DNA down-regulate this response. This raises the intriguing possibility that self DNA released by injured cells could down-regulate pathologic CpG-driven immune responses. While considerable additional research will be needed to evaluate this model, ongoing studies indicate that suppressive DNA can block the development of CpG-induced autoimmune disease in animal models (44, 45).

## Acknowledgments

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